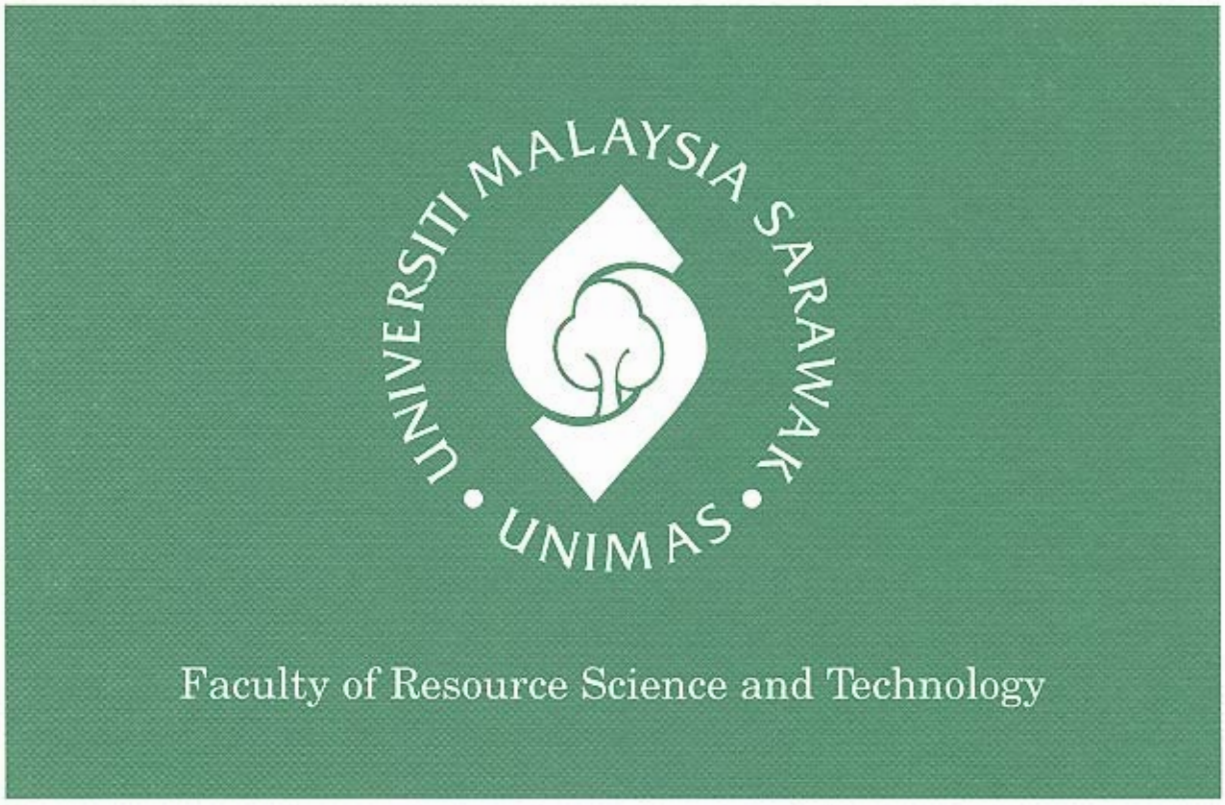

VIBRIO ALGINOLYTICUS FROM *ANADARA GRANULOSA*: SPECIES-SPECIFIC IDENTIFICATION BY TARGETING THE GENE OF NA-TRANSLOCATING NADH-QUINONE REDUCTASE COMPLEX, *(A-NQR)* AND RAPD-PCR FINGERPRINTING

Lai Che Ee

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Vibrio alginolyticus from *Anadara granulosa*: Species-specific Identification by Targeting the Gene of Na-translocating NADH-quinone reductase complex, (*A-Nqr*) and RAPD-PCR fingerprinting

Lai Che Ee

Resource Biotechnology Program
Faculty of Resource Science and Technology,
University Malaysia Sarawak

ABSTRACT

In this study, twenty-six of 60 sucrose fermenting *Vibrio spp.* isolates were identified as *Vibrio alginolyticus* by standard biochemical tests. Of the 26 *V. alginolyticus* isolates, 10 were further subjected to specific PCR using species-specific primer, targeting the *A-Nqr* gene for molecular confirmation. All 10 *V. alginolyticus* isolates produced amplified products of 407 base pairs (bp). Next, two short primers (Gen15008 and Gen15010) were used to obtain the genetic diversity of 26 *V. alginolyticus* isolates using random amplified polymorphic DNA (RAPD-PCR) analysis. The 26 isolates exhibited polymorphism with band ranging in size from 250 to 4500 bp. Dendrogram constructed from the RAPD profiles showed that the 26 isolates belong to 2 major clusters containing 6 and 20 isolates, respectively. The findings of this study showed that *V. alginolyticus* isolated from the *Anadara granulosa* sampled from 4 markets in Kuching are of multiple clones with a clonal diversity index of 0.791 (Gen15008) and 0.748 (Gen15010), and that the *A. granulosa* from different markets might be supplied from different geographical areas.

Keywords: *Vibrio alginolyticus; standard biochemical tests; specific PCR; RAPD PCR; Kuching.*

ABSTRAK

Daripada 60 pencilan, hanya 26 diklasifikasikan sebagai V. alginolyticus dengan ujian biokimia. Daripada 26 pencilan V. alginolyticus, 10 pencilan dipilih untuk pengeshan melalui tindak balas rantai polimerase spesifik dan pencetus spesifik, yang mengamplifikasikan gen A-Nqr. Kesemua 10 pencilan V. alginolyticus tersebut menghasilkan produk dengan saiz, 407 bp. Selepas itu, 2 pencetus pendek (Gen15008 dan Gen 15010) digunakan dalam tindak balas rantai polimerase RAPD untuk mendapatkan kepelbagaian genetik 26 pencilan V. alginolyticus tersebut. Kesemua pencilan V. alginolyticus itu menghasilkan kepelbagaian produk yang saiznya di dalam ringkungan antara 250 dan 4500 bp. Dendrogram yang dibina daripada profil RAPD menunjukkan bahawa 26 pencilan boleh dikumpulkan kepada 2 kumpulan yang utama yang terdiri daripada 6 dan 20 pencilan masing-masing. Keputusan-keputusan ini menunjukkan bahawa pelbagai klon V. alginolyticus telah dipencil daripada A. granulosa yang dikutip dari 4 market di Kuching dengan indeks kepelbagaian klon mencatat nilai 0.791 (Gen15008) dan 0.748 (Gen15010). Ini juga menerangkan kemungkinan A. granulosa di keempat-empat market ini ditangkap dari kawasan yang berlainan.

Kata kunci: *Vibrio alginolyticus; standard biochemical tests; specific PCR; RAPD PCR; Kuching.*

INTRODUCTION

Vibrio alginolyticus, a Gram negative halophilic curved rod, is a natural inhabitant of seawater (Zanetti *et al.*, 1999; Hormansdorfer *et al.*, 2000). *V. alginolyticus* is highly motile with a single polar flagellum (Kawagishi *et al.*, 1997; Kojima *et al.*, 1997) and is capable of causing wound, eye and ear infections in humans (Zanetti *et al.*, 1999; Elliot *et al.*, 2001). Consumption of raw seafood, especially oysters, or seafood with improper handling, increases the probability of *Vibrio*-associated gastroenteritis (Zanetti *et al.*, 1999). Furthermore, it is one of the pathogen in aquaculture, causing sea bream diseases like septicemia, hemorrhaging, dark skin and ulcers on the skin surface resulting in mortally of sea products and important economic losses (Balebona *et al.*, 1998; Hormansdorfer *et al.*, 2000; Zorrilla *et al.*, 2003).

Several studies have been done on *V. alginolyticus* isolated from different marine organisms (Balebona *et al.*, 1998) and from the seawater (Zanetti *et al.*, 1999; Hormansdorfer *et al.*, 2000). In a study by Balebona and colleagues (1998), the *in vivo* and *in vitro* pathogenic activities of whole cells and extracellular products of *V. alginolyticus* responsible for outbreaks that lead to mortality in cultured gilt-head sea bream were evaluated. Zanetti and colleagues (1999) differentiated *V. alginolyticus* strains isolated from Sardinian waters based on the specific amplification of genomic regions via PCR fingerprinting technique. PCR fingerprinting was also utilized in the same investigation and its superior discriminative power was proven for the differentiation of related *V. alginolyticus* strains, thus, revealing its potential application in epidemiological studies of this species (Zanetti *et al.*, 1999). Studies have also been done to determine the gene required for polar flagellum formation in *V. alginolyticus* (Kawagishi *et al.*, 1997). This study came with a conclusion that the highly motile *V.*

alginolyticus requires sodium for motility (Liu *et al.*, 1990; Asai *et al.*, 1999) and that the motility is strongly inhibited by phenamil (Kojima *et al.*, 1997).

In Malaysia, studies have been done on *Vibrio spp.* such as *V. cholerae* 01 and non-01, and *V. parahaemolyticus* (Son *et al.*, 1998; Son *et al.*, 2002), however, little is known about the presence of *V. alginolyticus* in *Anadara granulosa* (clam) for human consumption in Kuching, Sarawak. In this study, the presence of *V. alginolyticus* in twenty *A. granulosa* samples sampled between March 2003 to June 2003 and August 2003 were investigated. A total of sixty presumptive isolates were differentiated by standard biochemical tests, of which, twenty-six isolates were identified to be *V. alginolyticus*. In the studies of Hormansdorfer *et al.* (2000), standard biochemical tests were also being used to identify *V. alginolyticus* isolated from seawater aquaria. However, not all the standard biochemical tests performed by Hormansdorfer *et al.* (2000) were used in this study.

A pair of primers targeting the Na-translocating NADH-quinone reductase complex (*A-Nqr*) was designed with Primer 3 using the DNA sequence taken from the GenBank (A. No. AB008030). *A-Nqr* has been used in various studies to target the respective gene segments in *V. alginolyticus* encoding for Na-translocating NADH-quinone reductase complex (Beattie *et al.*, 1994; Hayashi *et al.*, 1994; Hayashi *et al.*, 1995; Nakayama *et al.*, 1998) which pumps sodium for its proton-powered flagella (Liu *et al.*, 1990). The expected fragment size was 407 bp. This technique is a rapid and reliable technique in species-specific identification of *V. alginolyticus* among the other bacteria that might be indistinguishable by the standard biochemical tests and the reproducibility of this specific PCR is high, thus, it is included in this study for molecular confirmation of *V. alginolyticus* isolates.

Randomly amplified polymorphic DNA (RAPD-PCR) is a technique widely used to obtain genetic fingerprints. RAPD-PCR was performed on all the isolates to compare genetic relatedness among the isolates. It had been widely used in genetic diversity studies involving *Vibrio spp.* other than *V. alginolyticus* such as *V. cholerae* (Son *et al.*, 2002), *V. parahaemolyticus* (Son *et al.*, 1998) and even on bacteria of other genera, *Escherichia coli* (Son *et al.*, 1999). Particular band patterns obtained from the RAPD-PCR performed on the samples were used for genetic relatedness calculation via the RAPDistance Package Version 1.04 (Armstrong, Australia). The Simpson Index of Diversity, one of many diversity indices used by biologists (Beals *et al.*, 1999) was used to measure the clonal diversity among the *V. alginolyticus* isolates. With this technology available, the genetic diversity within *V. alginolyticus* strains isolated from *A. granulosa* sampled from same and different markets in Kuching was obtained.

OBJECTIVES

1. To monitor the presence of *V. alginolyticus* in *A. granulosa* sold in Kuching, Sarawak.
2. To isolate *V. alginolyticus* from samples of *A. granulosa.*
3. To identify the *V. alginolyticus* isolates by performing standard biochemical tests.
4. To confirm the identity of the *V. alginolyticus* isolates by using a species-specific primer (*A-Nqr*) via specific PCR method.
5. To obtain genetic fingerprints of all the isolates of confirmed *V. alginolyticus* via randomly amplified polymorphic DNA (RAPD-PCR) technique for strains relatedness study.

MATERIAL AND METHODS

Materials

Sample collection, enrichment and isolation:

1. Alkaline Peptone Water (APW), pH 8.0 (Oxoid, UK)
2. LB (Luria Bertani) Broth (Fluka, Switzerland) + 3 % NaCl
3. Nutrient Agar (NA) (Oxoid, UK) + 3 % NaCl
4. Trypticase Soy Agar (TSA) (BBL, USA) + 3 % NaCl
5. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, UK)

Identification of bacterial strains (Standard Biochemical Tests):

1. Catalase Test Reagents –Hydrogen Peroxide, H_2O_2 3 % (Sigma, Germany)
2. Kligler Iron Agar (KIA) (Oxoid, UK)
3. ONPG Test Reagents (Oxoid, UK)
4. Secondary Selective Medium-purple agar (UNIMAS, Malaysia)
5. Simmons Citrate Agar (SCA) (Oxoid, UK)
6. Triple Sugar Iron Agar (TSI) (BBL, USA)
7. Tryptone Broth (1 %), without (0 % NaCl); or with 3, 6, or 8 % NaCl (Oxoid, UK)

DNA preparation (PCI extraction):

1. 1X TE Buffer
2. 1 liter ddH_2O
3. 10 % SDS (Bio-Rad, USA)
4. 20 mg/ml Proteinase K (Promega, USA)
5. 5 M NaAC (Hamburg, Germany)
6. Phenol/Chloroform/Isoamyl alcohol (25:24:1)
7. Isopropanol (R&M, UK)
8. 70 % Ethanol (Hamburg, Germany)

PCR amplifications:

1. 10X PCR Buffer (Promega, USA)
2. 15 mM $MgCl_2$ (Promega, USA)
3. 10 mM dNTPs (Promega, USA)
4. 5 pmol/µl Forward *A-Nqr* Primer (5'-CTG TGC AAT CTT CGG TGG TGT ATC-3') (MWG, Germany)
5. 5 pmol/µl Reverse *A-Nqr* Primer (5'-CAG AGA AGG GTC ATC GTT CAC AGA-3') (MWG, Germany)
6. 5 pmol/µl Random primer (Gen15008, 5'-GGAAGACAAC-3' & Gen15010, 5'-CCATTTACGC-3') (MWG, Germany)
7. Sterile MilliQ water
8. Taq DNA polymerase (Promega, USA)
9. Template DNA

Methods

Sample collection, enrichment, isolation and identification of bacterial strains

Twenty *A. granulosa* were sampled in the Kuching district, Sarawak, between March 2003 to June 2003 and August 2003. The entire interior contents (meat and fluid) of the *A. granulosa* (20 g) were transferred aseptically into 180 ml (1:10 dilution) of alkaline peptone water (APW) and homogenized. The homogenate were incubated for 8-16 hours at 37 °C. Longer period of incubation time was avoided to prevent background organisms from overwhelming the enrichment. After incubation, 10 µl of the upper layer was taken and streaked onto thiosulfate-citrate-bile salts-sucrose agar (TCBS), a selective media for *Vibrio spp.*, and incubated for 24 hours at 37 °C. On TCBS agar, *V. alginolyticus* form large and yellow colonies (Elliot *et al.*, 1994) due to fermentation of sucrose in TCBS. Three well-isolated yellow colonies were selected from each sample and were then subjected to standard biochemical tests for identification of *V. alginolyticus*. The tests included salt tolerance test, KIA and TSI agar tests, catalase test, SCA test, and ONPG test. The isolates were stored in nutrient agar and trypticase soy agar as stock cultures.

DNA preparation

Prior to amplification, chromosomal DNA of the isolates were extracted by the method of Ausubel *et al.* (1990). 1.5 ml of overnight bacteria culture in LB broth was centrifuged at 10,000 rpm for 1 minute and the supernatant was discarded. Then, 700 µl of TE buffer was

added to the tube, followed by vortexing the tube, then, 5 µl of proteinase K (25 mg/ml) and 10 µl of 10 % SDS were added. The solution was mixed gently and incubated at 60 °C for 20 minutes. After incubation, 500 µl of PCI mixture was added to the solution and brought to centrifuge at 12,000 rpm for 1 minute. After centrifugation, 200 µl upper layer of the supernatant was transferred into a new sterile tube and added with 200 µl 3 M K-Ac and 400 µl isopropanol. After well-mixed, the solution was left at room temperature for 5 minutes prior to centrifugation at 12,000 rpm for 7 minutes. The supernatant was discarded and the pellet was washed with 500 µl 70 % ethanol and further centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded after centrifugation and the pellet was air-dried at room temperature for 30 minutes after which was dissolved in 50 µl sdH_2O. The extracted chromosomal DNA was stored at –20 °C until used as template for specific PCR amplification of the *A-Nqr* gene and RAPD-PCR analysis.

Specific PCR amplification

A pair of 24-mer primers, *A-Nqr*F (5'-CTG TGC AAT CTT CGG TGG TGT ATC-3') and *A-Nqr*R (5'-CAG AGA AGG GTC ATC GTT CAC AGA-3'), targeting the Na-translocating NADH-quinone reductase complex (*A-Nqr*) in *V. alginolyticus* (Beattie *et al.*, 1994; Hayashi *et al.*, 1994; Hayashi *et al.*, 1995; Nakayama *et al.*, 1998) was designed with Primer 3 using the DNA sequence taken from the GenBank (A. No. AB008030). For the species-specific identification of *V. alginolyticus* among the twenty-six isolates, ten were randomly chosen for molecular confirmation using specific PCR method.

Table 1: Sequence and annealing temperature of species-specific primers for *V. alginolyticus*

Target gene *A-Nqr*		Primer sequence	Ta
Amplicon size	*A-Nqr*F	5'-CTG TGC AAT CTT CGG TGG TGT ATC-3'	65 °C
407 bp	*A-Nqr*R	5'-CAG AGA AGG GTC ATC GTT CAC AGA-3'	65 °C

Specific PCR reaction mixture of 25 µl volume reaction consists of:

Specific PCR reagent	Quantity per reaction
10X PCR buffer	: 2.5 µl
25 mM $MgCl_2$	: 2.0 µl
10 mM dNTPs	: 0.5 µl
5 pmol/µl *A-Nqr*F	: 1.0 µl
5 pmol/µl *A-Nqr*R	: 1.0 µl
Taq DNA polymerase	: 0.3 µl
Sterile MilliQ water	: 12.7 µl
Template DNA	: 5.0 µl
Total final volume	: 25.0 µl

The reactions were subjected to 30 temperatures cycles (Perkin Elmer 2400):

Step cycle	Temperature/Time
Initial denaturation	: 94 °C (3 min)
Denaturation	: 94 °C (1 min)
Annealing	: 65 °C (1 min) → 30 cycles
Elongation	: 72 °C (2 min)
Final elongation	: 72 °C (5 min)

The PCR products were visualized by running 5 µl product in a 1.0 % agarose gel in 1X TAE buffer. 1 kb DNA ladder (Promega, USA) and specific PCR pattern of a positive control, VA2B were included as size markers. The gel was electrophoresised at 70 V for 1 hour and thirty minutes. Upon staining with ethidium bromide (Sigma, Germany), the gel was visualized using U.V. transilluminator and captured using a polaroid camera.

RADP-PCR primers

Prior to RAPD-PCR amplification, ten randomly designed 10-mer oligonucleotide sets obtained from MWG Inc. (Germany) were screened for the best amplification for use in the final RAPD-PCR. The two best primers, namely, Gen15008 (5'-GGAAGACAAC-3') and Gen15010 (5'-CCATTTACGC-3') were selected for the final RAPD-PCR amplifications.

RAPD-PCR amplifications

Specific PCR reaction mixture of 25 µl volume reaction was as below:

Specific PCR reagent	Quantity per reaction
10X PCR buffer	: 2.5 µl
25 mM $MgCl_2$	: 2.0 µl
10 mM dNTPs	: 0.5 µl
5 pmol/µl Random Primer	: 2.0 µl
Taq DNA polymerase	: 0.3 µl
Sterile MilliQ water	: 15.7 µl
Template DNA	: 2.0 µl
Total final volume	: 25.0 µl

The reactions were subjected to 30 temperatures cycles (Perkin Elmer 2400):

Step cycle	Temperature/Time	
Initial denaturation	: 94 °C (3 min)	
Denaturation	: 94 °C (1 min)	→ 30 cycles
Annealing	: 36 °C (1 min)	
Elongation	: 72 °C (2 min)	
Final elongation	: 72 °C (5 min)	

The PCR products were visualized by running 5 µl product in a 1.0 % agarose gel in 1X TAE buffer. 1 kb DNA ladder (Promega, USA) was included as a size marker. The gel was electrophoresised at 70 V for 2 hours. Upon staining with ethidium bromide (Sigma, Germany), the gel was visualized using U.V. transilluminator and captured using a polaroid camera.

RAPD-PCR Data Analysis

The data obtained were analyzed using the RAPDistance Package Version 1.04 (Armstrong, Australia). The distance calculation was based on Dice (Nei & Li) and the dendrogram constructed was viewed using Adobe Photoshop 6.0. Genetic diversity of isolates was then calculated based on the Simpson Index of Diversity.

RESULTS

Table 2: Nomenclature of presumptive isolates with its month source and origin of sampling.

PLACE	MONTH	YELLOW COLONY ISOLATES	PLACE	MONTH	YELLOW COLONY ISOLATES
7th mile	March	YI	Green Road	March	Y25
		Y2			Y26
		Y3			Y27
	April	Y4		April	Y28
		Y5			Y29
		Y6			Y30
	May	Y7		May	Y31
		Y8			Y32
		Y9			Y33
	June	Y10		June	Y34
		Y11			Y35
		Y12			Y36
	August	Y49		August	Y55
		Y50			Y56
		Y51			Y57
3rd mile	March	Y13	Matang	March	Y37
		Y14			Y38
		Y15			Y39
	April	Y16		April	Y40
		Y17			Y41
		Y18			Y42
	May	Y19		May	Y43
		Y20			Y44
		Y21			Y45
	June	Y22		June	Y46
		Y23			Y47
		Y24			Y48
	August	Y52		August	Y58
		Y53			Y59
		Y54			Y60

Table 3: Results of sixty presumptive isolates towards standard biochemical tests.

SS/MM	Isolate	T1N0	T1N3	T1N6	T1N8	KIA	TSI	SCA	Catalase	ONPG	Purple agar
S3	Y1	-	+	-	+	K/A	A/A	-	+	-	+
S3	Y2	-	+	+	+	K/A	K/A	-	+	-	-
S3	Y3	-	+	+	+	K/A	-	-	-	-	-
S4	Y4	-	+	+	+	K/A	A/A	-	+	-	+
S4	Y5	+	+	+	-	K/A	K/A	-	+	-	-
S4	Y6	-	+	+	+	K/A	A/A	-	+	-	-
S5	Y7	-	+	+	-	K/A	A/A	-	+	-	-
S5	Y8	-	+	+	+	K/A	A/A	-	+	-	-
S5	Y9	-	+	+	+	K/A	K/A	-	+	-	-
S6	Y10	-	+	+	+	K/A	K/A	+	+	-	-
S6	Y11	-	+	+	+	K/A	A/A	-	+	-	+
S6	Y12	+	-	-	-	K/A	K/A	+	+	-	-
T3	Y13	-	+	+	-	K/A	A/A	-	-	-	-
T3	Y14	-	+	-	-	K/A	A/A	-	-	-	-
T3	Y15	-	+	-	-	K/A	A/A	-	-	-	-
T4	Y16	-	+	+	+	K/A	K/A	-	+	-	-
T4	Y17	-	+	+	+	K/A	A/A	-	+	-	+
T4	Y18	-	+	+	+	K/A	K/A	+	+	-	-
T5	Y19	-	+	+	+	K/A	K/A	-	+	-	-
T5	Y20	-	+	+	-	K/A	A/A	+	-	-	-
T5	Y21	-	+	+	+	K/A	A/A	-	+	-	+
T6	Y22	-	+	+	+	K/A	K/A	-	+	-	-
T6	Y23	+	+	-	-	K/A	K/A	-	+	-	-
T6	Y24	-	+	+	+	K/A	A/A	-	+	-	+
G3	Y25	-	+	-	+	K/-	A/A	-	+	-	+
G3	Y26	-	-	+	+	K/-	K/A	-	+	-	+
G3	Y27	-	+	+	+	K/-	A/A	-	+	-	+
G4	Y28	-	+	+	+	K/-	A/A	-	+	-	+
G4	Y29	-	+	-	-	K/-	K/A	-	+	-	+
G4	Y30	-	+	+	+	K/-	A/A	-	+	-	+
G5	Y31	-	+	+	+	K/-	A/A	-	+	-	+
G5	Y32	-	+	+	+	K/-	A/A	-	+	-	+
G5	Y33	-	+	+	-	-	A/A	-	-	-	-
G6	Y34	-	+	+	-	-	A/A	-	-	-	-
G6	Y35	-	+	+	-	-	A/A	-	-	-	-
G6	Y36	-	+	+	+	K/-	A/A	-	+	-	+
M3	Y37	+	-	+	-	K/-	A/A	-	+	-	-
M3	Y38	-	+	+	+	K/-	A/A	-	+	-	+
M3	Y39	-	+	+	+	K/-	A/A	-	+	-	+
M4	Y40	-	+	-	-	K/-	K/A	-	-	-	-
M4	Y41	-	+	+	+	K/-	A/A	-	+	-	+
M4	Y42	-	+	+	-	-	K/A	-	-	-	-
M5	Y43	-	+	+	-	K/-	K/A	-	-	-	-
M5	Y44	-	+	+	+	K/-	A/A	-	+	-	+
M5	Y45	-	+	-	-	K/-	A/A	-	-	-	-
M6	Y46	-	+	-	+	K/-	A/A	-	+	-	+
M6	Y47	-	-	-	-	-	-	-	-	-	-
M6	Y48	-	-	-	-	-	-	-	-	-	-
S8	Y49	-	+	+	+	K/-	A/A	-	+	-	+
S8	Y50	-	+	+	+	K/-	A/A	-	+	-	+
S8	Y51	-	+	+	-	-	A/A	-	-	-	-
T8	Y52	+	+	-	-	-	A/A	-	-	-	-
T8	Y53	-	+	+	+	K/-	A/A	-	+	-	+
T8	Y54	-	+	+	+	K/-	A/A	-	+	-	+
G8	Y55	-	+	+	+	K/-	A/A	-	+	-	+
G8	Y56	-	+	+	-	K/-	K/A	-	+	-	+
G8	Y57	-	+	+	+	K/-	A/A	-	+	-	+
M8	Y58	-	+	+	+	K/-	A/A	-	+	-	+
M8	Y59	-	+	-	-	K/-	K/A	-	+	-	+
M8	Y60	-	+	+	+	K/-	A/A	-	+	-	+

Note: SS, source: S, 7th mile market; T, 3rd mile market; G, Green Road market; M, Matang market.
MM, month: 3, March; 4, April; 5, May; 6, June; 8, August.
Slant/butt: K, alkaline (pink); A, acid (yellow); +, positive reaction; -, negative reaction.

Figure 1: Formation of colonies on TCBS
Note: Yellow colonies isolated as presumptive isolates

Figure 2: Triple Sugar Iron Agar
Note: Uninoculated (left); positive result [A/A (slant/butt)], showing presence of *V. alginolyticus* (right).
K, alkaline (pink); A, acid (yellow).

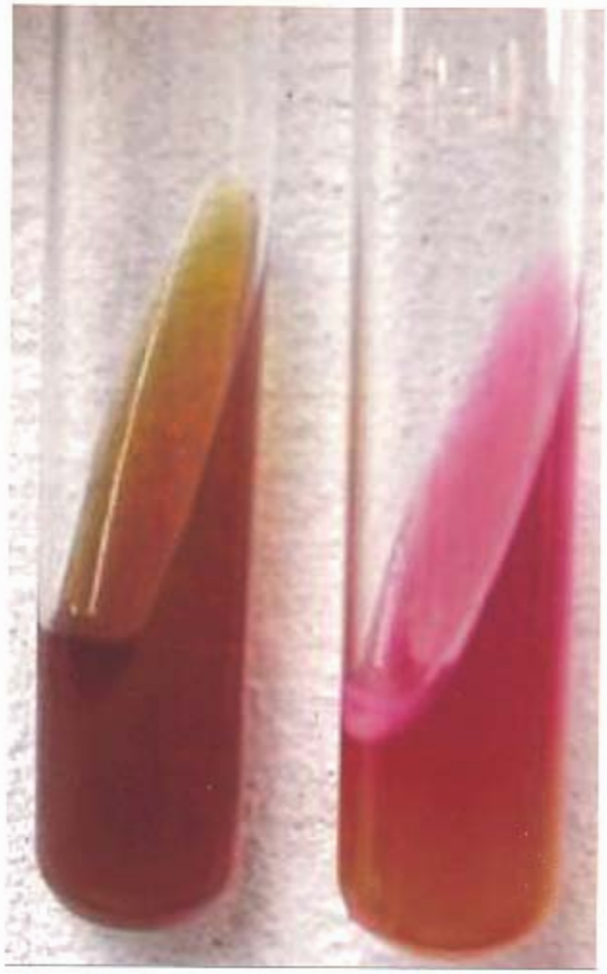

Figure 3: Kligler Iron Agar

Note: Uninoculated (left); positive result [K/A (slant/butt)], showing presence of *V. alginolyticus* (right)
K, alkaline (pink); A, acid (yellow).

Figure 4: Salt tolerance test

Note: Turbidity as positive reaction: T_1N_0, negative growth; T_1N_3, T_1N_6 and $T_1N_{8.5}$, positive growth (left to right)

Figure 5: Catalase test
Note: catalase-positive (left), catalase-negative (right).
Taken from: Cappuccino *et al.*, (1992)

Figure 6: Simmons Citrate Agar
Note: Uninoculated (left); negative result (right) indicating the presence of *V. alginolyticus*

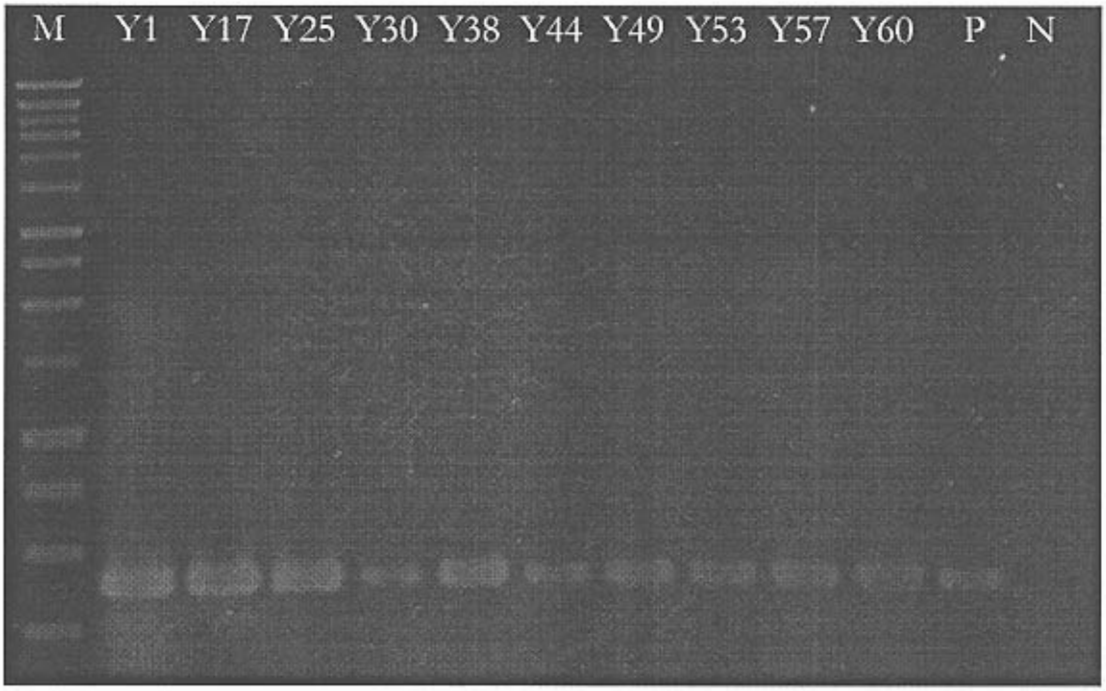

Figure 7: Specific PCR pattern obtained using primer *A-Nqr* targeting the Gene of Na-translocating NADH-quinone reductase complex for *V. alginolyticus* strains isolated from *A. granulosa*.

Note: Lane 1 (M): 1 kb DNA ladder (Promega, USA); lanes 2-11: representatives of the 10 specific PCR pattern obtained; lane 12 (P): specific PCR pattern of a positive control, VA2B; lane 13 (N): negative control.

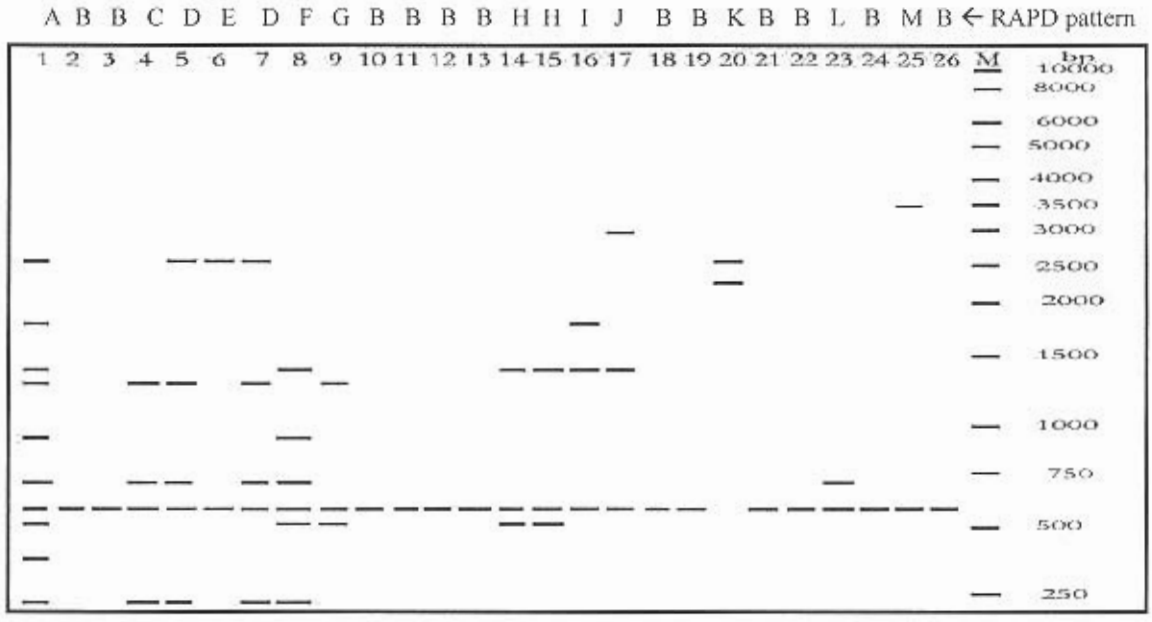

Figure 8: Digitalised RAPD patterns obtained using primer Gen15008 for twenty-six *Vibrio alginolyticus* isolates.

Note: Lanes 1-26: representatives of the 26 RAPD patterns obtained, Y1, Y4, Y11, Y17, Y21, Y24, Y25, Y27, Y28, Y30, Y31, Y32, Y36, Y38, Y39, Y41, Y44, Y46, Y49, Y50, Y53, Y54, Y55, Y57, Y58, Y60, respectively; lane M: 1 kb DNA ladder (Promega, USA). Letters across the top indicates the RAPD patterns.

Figure 9: Digitalised RAPD patterns obtained using primer Gen15010 for twenty-six *Vibrio alginolyticus* isolates.

Note: Lanes 1-26: representatives of the 26 RAPD patterns obtained, Y1, Y4, Y11, Y17, Y21, Y24, Y25, Y27, Y28, Y30, Y31, Y32, Y36, Y38, Y39, Y41, Y44, Y46, Y49, Y50, Y53, Y54, Y55, Y57, Y58, Y60, respectively; lane M: 1 kb DNA ladder (Promega, USA). Letters across the top indicates the RAPD patterns.

Table 4: RAPD-PCR patterns using primer Gen15008 and Gen15010 for *V. alginolyticus* isolates.

Isolate	*A. granulosa* Source (Market)	RAPD Patterns Gen15008	Gen15010
Y1	7th Mile	A	A
Y4	7th Mile	B	A
Y11	7th Mile	B	A
Y17	3rd Mile	C	B
Y21	3rd Mile	D	B
Y24	3rd Mile	E	B
Y25	Green Road	D	B
Y27	Green Road	F	B
Y28	Green Road	G	A
Y30	Green Road	B	E
Y31	Green Road	B	C
Y32	Green Road	B	C
Y36	Green Road	B	C
Y38	Matang	H	D
Y39	Matang	H	A
Y41	Matang	I	A
Y44	Matang	J	E
Y46	Matang	B	C
Y49	7th Mile	B	C
Y50	7th Mile	K	A
Y53	3rd Mile	B	A
Y54	3rd Mile	B	A
Y55	Green Road	L	C
Y57	Green Road	B	C
Y58	Matang	M	A
Y60	Matang	B	B

Table 5: Number of isolates with particular RAPD-PCR pattern using primer Gen15008

Primer Gen15008		
RAPD-PCR pattern	number of isolates, n	n(n-1)
A	1	1(0)=0
B	12	12(11)=132
C	1	1(0)=0
D	2	2(1)=2
E	1	1(0)=0
F	1	1(0)=0
G	1	1(0)=0
H	2	2(1)=2
I	1	1(0)=0
J	1	1(0)=0
K	1	1(0)=0
L	1	1(0)=0
M	1	1(0)=0
Total (N)	26	136

Calculation 1: The Simpson Index of Diversity of isolates For Gen15008

- $D = \frac{\Sigma\, n(n-1)}{N(N-1)}$

  $= \frac{136}{26(25)}$

  $= 0.2092308$

  $= 0.209$ (3 decimal point)

- The Simpson index of diversity = 1-D

  $= 1 - 0.209$

  $= 0.791$

Table 6: Number of isolates with particular RAPD-PCR pattern using primer Gen15010

Primer Gen15010		
RAPD-PCR pattern	number of isolates, n	n(n-1)
A	10	10(9)=90
B	6	6(5)=30
C	7	7(6)=42
D	1	1(0)=0
E	2	2(1)=2
Total (N)	26	164

Calculation 2: The Simpson Index of Diversity of isolates for Gen15010

- $D = \frac{\Sigma\, n(n-1)}{N(N-1)}$

  $= \frac{164}{26(25)}$

  $= 0.2523077$

  $= 0.252$ (3 decimal point)

- The Simpson index of diversity = 1-D

  $= 1 - 0.252$

  $= 0.748$

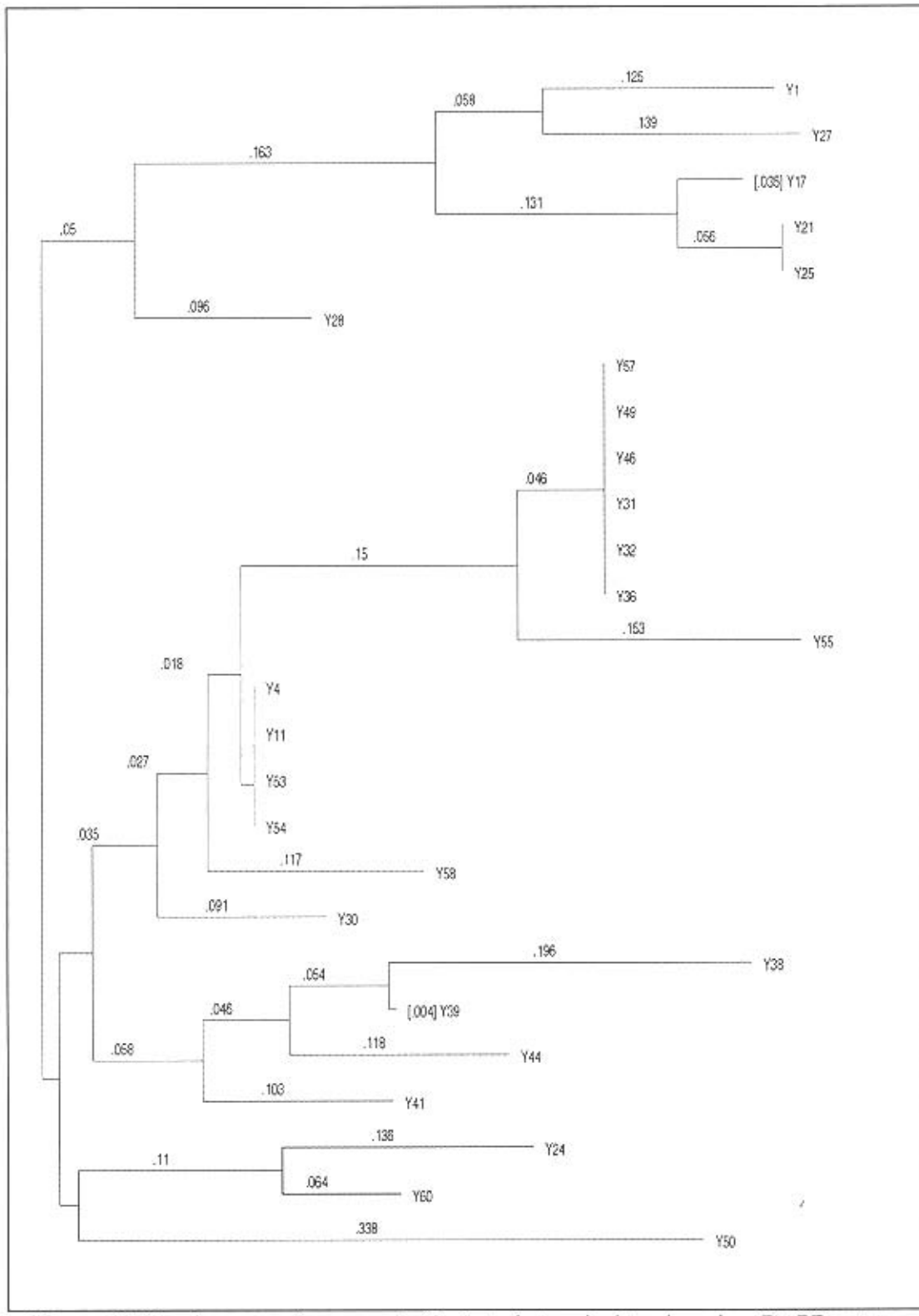

Figure 10: Dendrogram of twenty-six *V. alginolyticus* isolates based on RAPD patterns.